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**PERINATAL REGENERATIVE MEDICINE
- DEVELOPMENT OF AUTOLOGOUS
CONSTRUCTS FOR SOFT TISSUE
DEFECTS**

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Perinatal Regenerative Medicine
- development of autologous constructs for soft tissue defects

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family with love

ABSTRACT

Disrupted organogenesis leads to permanent malformations that may require surgical correction. Affected new-borns without spare tissue for the primary closure are dependent of other solutions. Because of the perinatal tissue shortage, synthetic or biological prosthetic materials are being used, which may be associated with complications. The progress in cell-based therapies makes autologous tissue engineering a rational alternative to current treatments, and infants with prenatally diagnosed soft tissue defects should benefit from having autologous tissue readily available for surgical implantation in the perinatal period. The aim of this thesis was to investigate cell and tissue sources harvested pre- or postnatally for development of autologous constructs that could be used in the correction of congenital soft tissue defects. We sought a reliable and clinical feasible isolation and culture method, a cell or tissue source with minimal or no donor site morbidity and high yield of viable cells with minimal handling or additives. Amniotic fluid (AF) can easily be obtained during routine amniocentesis for foetal karyotyping and in study I, we investigated the cell content in AF and the isolation and expansion potential of AF cells (AFC). The study showed that AF displayed large donor variability with varying isolation and expansion success of the AFC, and therefore it may not be the best source for autologous cell therapy. A small tissue biopsy could also be obtained during amniocentesis, and in study II we investigated foetal subcutaneous cells (fSC) as cellular source for tissue engineering. We also combined the expanded fSC with two different matrices; cell free small intestine submucosa (SIS) and collagen gel combined with poly(ϵ -caprolactone). The cells showed low adherence to SIS and did not migrate deep into the matrix. However, in collagen gels the cells migrated into the gel and proliferated with sustained viability for up to 8 weeks. The fSC demonstrated a high proliferative capacity and favourable characteristics for the preparation of autologous tissue transplants before birth. In study III we explored two postnatal tissue sources; the amnion membrane from term placenta and a gel made of plasma rich in growth factors (PRGF) from umbilical cord blood (UCB). The amnion and the gel were combined with SIS to form constructs. The constructs do not include cell expansion in vitro, can be made promptly after birth with minimal handling and can be stored for to 2-3 weeks if the surgical correction is performed later. Our study showed that amnion and UCB are promising sources for production of autologous grafts for the correction of congenital soft tissue defects. The main conclusion of this thesis is that depending on the timeframe from diagnosis to birth and type, size and severity of the defect, either 2nd trimester fSC, term amnion or PRGF gel are the preferable perinatal sources for autologous tissue engineering and correction of the congenital soft tissue defect.

LIST OF SCIENTIFIC PAPERS

- I. Åsa Ekblad,** Hong Qian, Magnus Westgren, Katarina Le Blanc, Magdalena Fossum, and Cecilia Götherström.
Amniotic fluid – a source for clinical therapeutics in the newborn?
Stem Cells and Development. 2015 Jun 15;24(12):1405-14. PMID 25668721
- II. Åsa Ekblad,** Magnus Westgren, Magdalena Fossum and Cecilia Götherström
Fetal subcutaneous cells have potential for autologous tissue engineering.
Journal of Tissue Engineering and Regenerative Medicine, 2018;1–9. PMID 29327490
- III. Åsa Ekblad,** Magdalena Fossum and Cecilia Götherström
Soft tissue repair with easy-accessible autologous newborn placenta or umbilical cord blood in severe malformations: A primary evaluation
Stem Cells International, Volume 2017 (2017): 1626741. PMID 29403534

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LIST OF ABBREVIATIONS

α -SMA	α -smooth muscle actin
aBM-MSC	Adult bone marrow mesenchymal stromal cells
ADC	Amnion-derived cells
AF	Amniotic fluid
AFC	Amniotic fluid cells
Amnion-SIS	Amnion and small intestine submucosa construct
CDH	Congenital diaphragmatic hernia
CFSE	Carboxyfluorescein succinimidyl ester
CFU-F	Colony-forming unit fibroblasts
Chang	Minimum essential medium alpha and 20 % Chang medium
CM	Conditioned medium
CM-fSC	Conditioned medium from foetal subcutaneous cells
cpm	Counts per minute
CVB	Chorionic villi biopsies
DMEM	Dulbecco's modified eagle medium
DMEM-EC	Epithelial cell culture medium
DMEM-F12	Dulbecco's modified eagle medium/Nutrient mixture F-12
E	Embryonic day
EC	Epithelial cells
ECM	Extra cellular matrix
FCS	Foetal calf serum
Fetoscopy	Examination of a foetus within the uterus by using an endoscope
Fibr.	Fibroblast cells
fSC	Foetal subcutaneous cells
GMP	Good Manufacturing Practice
GvHD	Graft versus host disease
GW	Gestational week
hESC	Human embryonic stem cell
HTX	Hematoxylin
HUVEC	Human umbilical vein endothelial cells
MEM-alpha	Minimum essential medium alpha
MLC	Mixed lymphocyte culture
MMC	Myelomeningocele
MNC	Mononuclear cells

MSC	Mesenchymal stromal cells
NTD	Neural tube defect
P	Passage
PCK	Pan-cytokeratin
PCL	Poly(ϵ -caprolactone)
PD	Population doublings
PDT	Population doubling time
PFA	Paraformaldehyde
PPGF	Plasma poor of growth factors
PRGF	Plasma rich in growth factors
PRGF-SIS	Construct of mononuclear cells enriched plasma rich in growth factors and small intestine submucosa
SB	Spina bifida
SIS	Small intestine submucosa
TE	Tissue engineering
UC	Umbilical cord
UCB	Umbilical cord blood
UCB-MSC	Umbilical cord blood mesenchymal stromal cells
UC-MSC	Umbilical cord mesenchymal stromal cells

1 INTRODUCTION

Being born with a congenital soft tissue malformation might mean that you have a lifelong relationship with your defect, corrected or not, whether it is a small scar or a condition restricting your personal daily performance. During childhood, it may lead to repeat hospital visits with regular examinations, limiting your presence at school, or a feeling of discomfort when sharing dressing room at gymnastic class. It may also affect the social-emotional well-being of the child.

The focus of this thesis is on congenital soft tissue malformations, defects with absent or incompletely formed tissues, involving the tissue surrounding the abdominal cavity and the spine. There are different options available for the closure of such defects, but in major malformations there are currently limited alternatives.

1.1 EMBRYONIC DEVELOPMENT AND ORGANOGENESIS

The human embryonic structural development from fertilized egg to a developing foetus is chronologically organized in the Carnegie stages (Figure 1) [1]. For example; Carnegie stage 1 includes the fertilized egg at embryonic day one (E1). Implantation occurs in stage 5, (E7-12) and gastrulation at stage 6 and 7 (E13-17). Organogenesis starts at Carnegie stage 9 (E19-21) with ectodermal neural tube formation and mesodermal segmentation for early somite formation finalizing in stage 23 (E56-60) when all primitive organs are formed [2]. Thereafter the embryo is considered as a foetus. Alongside with the embryogenesis there is extraembryonic tissue formation, essential for embryonic survival [3]. The amniotic mesoderm and yolk sac are the first extraembryonic tissues that are formed. The amniotic mesoderm is formed at E6 and is the precursor to the chorion and amnion membranes forming the fluid filled amniotic cavity that protects the foetus against desiccation and mechanical stress [3]. The primary yolk sac is formed at E7-12 and in combination with the allantois it acts as a primitive placenta supporting embryonal growth during organogenesis and providing the embryo with nutrients and later primitive hematopoietic functions [4, 5]. The allantois develops from the secondary yolk sac that fuses with the chorion at E13-14, and in it umbilical vessels are generated that induce placental development [6] for foetal-maternal circulation.

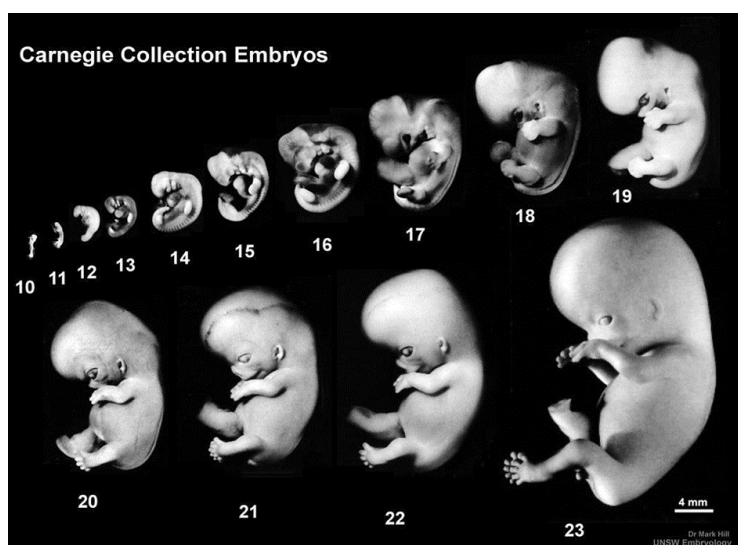


Figure 1. Illustration of Carnegie stages 10-23 (E22-60) of the developing embryo when the organogenesis occurs.

Illustration reproduced with permission from fertilitypedia.org.

1.2 CONGENITAL MALFORMATIONS

Human organogenesis occurs during approximately 36 days, but already at the end of Carnegie stage 14 (E32) most primitive organs can be identified, making E19-E32 the most vulnerable period during organogenesis [7], and any aberration during this time period leads to consequences for the remaining foetal development. Disrupted organogenesis leads to permanent congenital malformations [8]. The prevalence of significant congenital malformations is 2-3 % of all births and the etiologic classification divides the causes into two categories; primary (genetic: chromosomal abnormalities, mutations or polygenic causes), and secondary (environmental: e.g. toxins, viruses and bacteria, chemicals, drugs and maternal conditions such as hyperglycaemia or other metabolic conditions). Most malformations are described as complex, meaning that both genetics and environment causes the malformation and genetics lead to an increased susceptibility to develop malformations [9]. Congenital malformations are the leading cause of neonatal death but are also associated with perinatal morbidity and responsible for a significant part of paediatric hospital admissions and costs [10-12]. The anomalies are often detected at prenatal screenings during pregnancy and the pregnant woman may be offered amniocentesis or other prenatal diagnostics to exclude comorbidities or chromosomal abnormalities. Antenatal diagnosis is important and permits time for adequate parental counselling, plan for the delivery site and preparation of the specialist team at a neonatal care unit [13], all which aim at improving the health and clinical outcome of the mother and baby. Soft tissue malformations involving the abdominal cavity and the spine are most often surgically corrected within hours to days after birth depending on the type, size and severity of the defect. Major soft tissue defects may need replacement of the missing tissue in order to close the defect without causing high abdominal pressure and/or respiratory problems, for these cases, different treatment options are available.

1.2.1 Congenital soft tissue defects

Congenital malformations can occur in all organ systems such as the heart, liver, nervous system, bones and urinary tract [8]. In this thesis the focus is on soft tissue defects, mainly skin, muscle and tendon and in particular soft tissue malformations of the abdominal cavity and the spine. The methods described can be applicable for other clinical conditions involving soft tissue defects, but we chose to focus on few, but severe conditions that might give rise to large scar formations.

1.2.1.1 *Spina Bifida*

Spina Bifida (SB) is a neural tube defect (NTD) with improper neural tube formation, leading to incomplete closure of the vertebrae and meninges protecting the spinal cord. There are two main types of spina bifida; closed-lesion SB (*spina bifida occulta*) and open-lesion SB (*spina bifida aperta*). SB occulta is the mildest form (Figure 2A), where the lesion is unexposed and often asymptomatic. SB aperta is the severe form where the neural tissue is exposed with or without a protruding sac as a result from the incomplete formation of the vertebrae.

Meningocele is one form of SB aperta, where the spinal cord is not included in the hernia (Figure 2B). *Myelomeningocele* (MMC) is the severe form of SB aperta where the meninges herniate with protrusion of a wedged spinal cord (Figure 2C) [14, 15]. Despite a decrease in the prevalence of SB by the introduction of dietary folic acid fortifications taken by women before conception [16] there is a rather high prevalence of MMC; about 1500 diagnosed children are born annually in USA. In Europe there is no mandatory dietary folic acid fortifications and the prevalence for SB is 8.16/10 000 new-borns annually (in Sweden 2.9) [17].

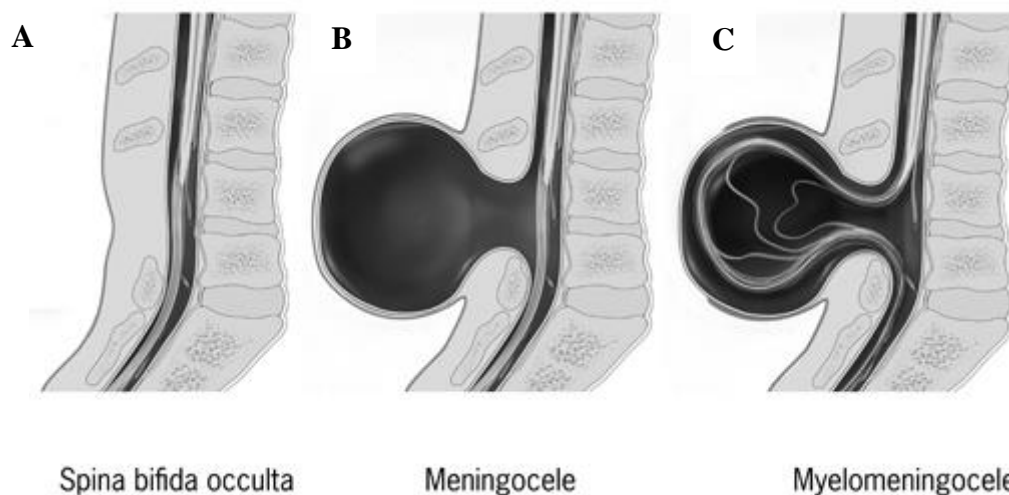


Figure 2. Illustrations of different types of spina bifida with the least severe form spina bifida occulta to the left and to the right myelomeningocele, the most severe form of spina bifida aperta.

Illustration reproduced with permission from Centers for Disease Control and Prevention, National Center on Birth Defects and Developmental Disabilities.

1.2.1.2 Abdominal wall defects

Gastroschisis and omphalocele are the most common congenital abdominal wall defects and are sometimes associated with co-morbidities, but the pathogenesis is not fully known [18, 19]. Gastroschisis is a full-thickness abdominal wall defect, most commonly located on the right side of the umbilicus, leading to foetal intestine protrusion and exposure to the amniotic fluid throughout the remaining pregnancy (Figure 3A) [20]. Omphalocele is a centrally located defect with herniated abdominal organs in a sac connected to the umbilical cord (Figure 3B) [21]. Gastroschisis and omphalocele are classified by the severity of the conditions (simple or complex gastroschisis and small, giant, or ruptured omphalocele).

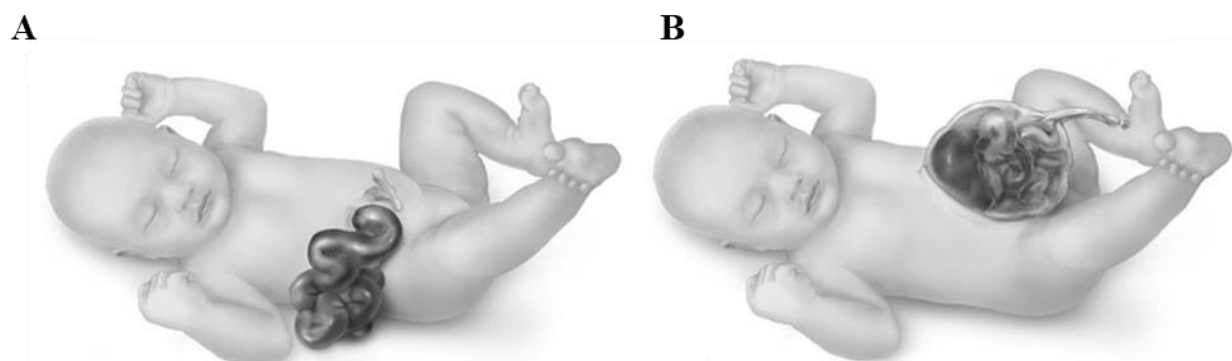


Figure 3. Drawings of babies with **A)** gastroschisis and **B)** omphalocele.

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1.2.1.3 Congenital diaphragmatic hernia

Congenital diaphragmatic hernia (CDH) is a rare but clinical complex deformity with high mortality rate and severe co-morbidities [22, 23]. The underlying pathogenesis of CDH is believed to be failed closure of the pericardioperitoneal canals. The open canals lead to abdominal content intrusion into the thorax with following compression of the primitive foetal

lung [24] and interference of the further lung development [25] (Figure 4). CDH is classified with a standardised severity grading from A (smallest) to D (largest) depending on size of the hernia [23].

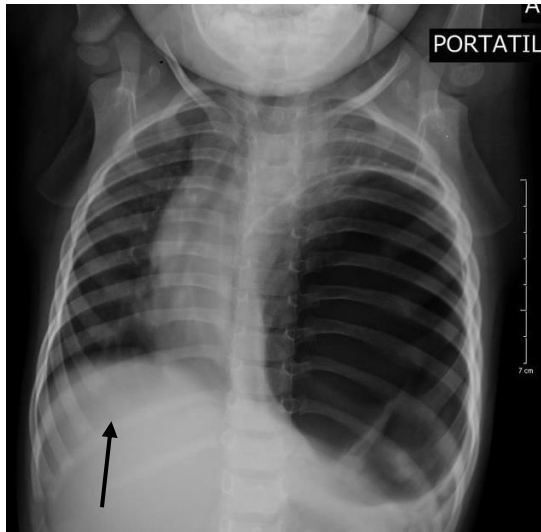


Figure 4. Chest radiograph of a thorax demonstrating a diaphragmatic Bochdalek hernia. Note the large air filled intestinal matter filling up the left thorax cavity and compressing the left lung parenchyma cranially and pushing the mediastinum to the right thoracic cavity (Black arrow). Picture reproduced with permission from Radiopaedia.org.

1.2.2 Current treatment strategies for major soft tissue defects

In all minor uncomplicated cases of soft tissue defects, the defect is primary closed by approximating surrounding tissues (Figure 5 A), but larger defects are more demanding as these may be difficult to close without tensions in the closure area.

There are currently several different approaches of closure depending on severity and type of defect. The categories are either the primary closure with or without relaxing incisions (Burow's triangle) or different variants of rotational tissue flaps with or without muscle tissue (fasciocutaneous and myocutaneous flaps). Closure with tissue flaps leads to increased wound areas and thereby larger scars (Figure 5 B-D). When there is insufficient proximal tissue, autologous tissue (autograft) can be retrieved from a healthy donor site but that leads to two sites of healing and donor site morbidity (Figure 5 E) [26]. There are also limitations depending of the needed graft size. The solution can then be to mesh the donor tissue to enlarge its size to enable greater defect area coverage. This reduces the donor site area and increases the graft area but the gaps in the graft tissue mesh might lead to some scar formation that may be more severe compared to tissue flaps [27].

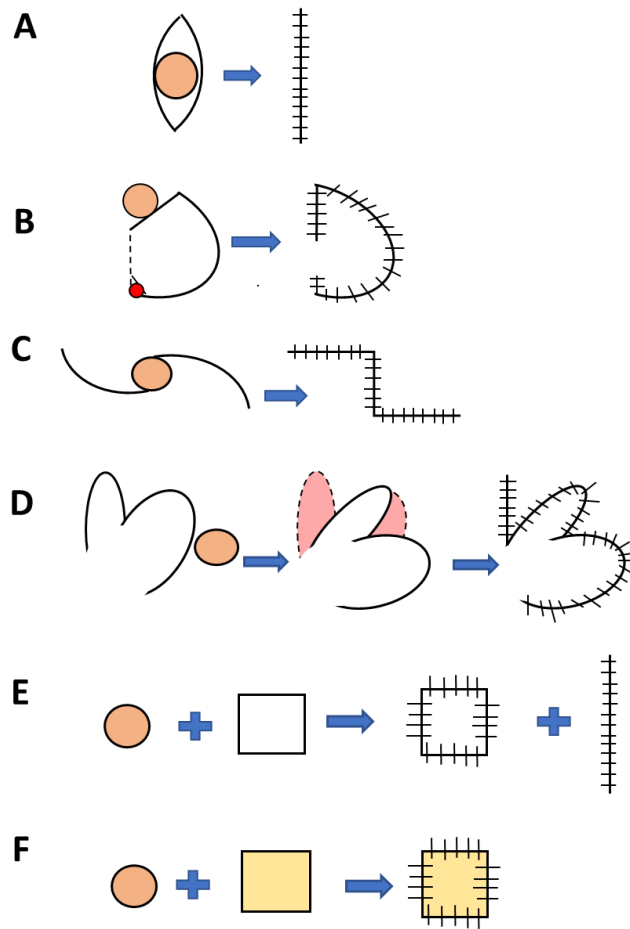


Figure 5. Illustrations of different closure techniques for soft tissue defects. **A)** Primary closure with or without relaxing incisions. **B)** Single rotational flap. Note the so-called Burow's triangle (marked as a red dot) for the reduction of tensions. **C)** Double or multiple rotational flaps. It can consist of skin and muscle flaps. **D)** Bi-lobed flap, for reduction of tensions and increasing the rotation ability. **E)** Closure with autograft from a donor site and **F)** Closure with an engineered graft (illustrated as a yellow square). ● marks the defect to be covered. Cartoon made by the author.

Another advantage with tissue flaps is the maintained blood supply whilst an autograft needs to regenerate new vessels in order to survive, events that takes a few days [28]. If insufficient autologous tissue is available, or to avoid tensions and tearing in the closure area, tissue substitutes from different sources can be used (Table 1).

Allogeneic tissue grafts eliminate donor site morbidity but can induce a recipient immune response with eventual graft degradation [28] and rejection with subsequent necrosis and other related complications [29]. Synthetic materials can also be used, but they lack plasticity, can be rejected and might need replacement after recipient growth [30]. Decellularisation of allogeneic and xenogeneic grafts reduces the risks for adverse events and the cell-free remaining extracellular matrix (ECM) can be repopulated by the recipient cells during the regenerative period [31]. Acellular ECM products derived either from cadaveric human or animal tissue supports tissue remodelling and neovascularisation, have lower rate of infections compared to synthetic materials and advantages in high risk contamination areas [32-34].

Table 1. Comparison of the most common graft sources.

Graft source	Definition	Advantages	Disadvantages
Autologous	Tissue or cells derived from the recipient itself.	Recognised as "self" and will not induce an immune response or be rejected.	Donor site morbidity and limited access of tissue.
Allogeneic	Tissue or cells derived from same species.	No donor site morbidity or major accessible size restrictions.	Recognised as foreign, might induce immune response, graft versus host-disease (GvHD) or get rejected.
Xenogeneic	Tissue or cells derived from animals.	No donor site morbidity and almost limitless accessibility.	Recognised as foreign, might induce immune response or get rejected. There is a concern for transmitted animal-related diseases.
Synthetic	Various artificial tissue substitutes.	Non-immunogenic materials with selected mechanical strength.	Some synthetic materials are non-absorbable and do not grow with the recipient child and therefor might need to be replaced.

1.2.2.1 Spina bifida

There are both pre- and postnatal treatment options for MMC. Prenatal surgical interventions that may preserve neurological functions have long been a controversial treatment modality due to risks for premature birth and unknown postnatal effects on outcome. This, in combination with the complexity of the intervention, prenatal correction of the exposed spinal hernia is only performed in a few highly specialized centers in the world [35]. Closure of the spinal defect involves multilayer closure of the dura, muscular fascia and finally the skin [36].

1.2.2.2 Abdominal wall defects

In the case of a clinical complex gastroschisis or giant omphalocele the tissue flaps can be combined with a prosthetic silo for a staged, progressive closure [21, 37]. Abdominal wall high risk defects not amendable for conventional closure, including ischemic bowel or perforated intestines, requires a synthetic or biological prosthesis for closure or cover of the defect area until stabilisation [38].

1.2.2.3 CDH

In most centers performing prenatal surgery for CDH, the treatment is based on enhancing lung tissue growth by plugging the trachea, not correcting the diaphragmatic defect [39]. CDH can also be corrected prenatally but no advantages of the clinically outcome compared to postnatal treatment has been reported [40]. Postnatally, small CDH defects (class A) can be primary repaired with proximal tissue, while larger defects (class D) also needs tissue replacements for the correction [23]. The replacements used for the correction of severe congenital malformations may consist of synthetic materials (e.g. Gore-Tex[®]) or acellular ECM-derived products such as human dermis (e.g. Alloderm[®]), bovine dermis (e.g. Permacol[®]), porcine dermis (e.g. Strattice[™]) or porcine small intestine submucosa (SIS, e.g. Biodesign[®], former Surgisis[®]) [8, 32, 38].

1.2.3 Treatment drawbacks

The drawback with current closure methods are 1) the scar size from tissue flaps, 2) the severity of the scar formed, donor site morbidity and limited tissue availability from autologous grafts and 3) potential rejection or other complications because of allogeneic or synthetic grafts. Another drawback with cell-free grafts is that the grafted area needs to be repopulated with innate cells. Pre-populating grafts with cells has been shown to enhance graft integration and restoration of host tissue and to reduce scar formation and bacterial infections which make tissue engineering a promising prospective [41, 42].

1.3 TISSUE ENGINEERING

Tissue engineering (TE), is an interdisciplinary area within the field of regenerative medicine, combining engineering, biology, material science and surgery [43, 44]. The concept was first established in the 1980's [45] with the aim to mimic organogenesis and to produce living tissue substitutes [46]. The most common approach of TE involves a combination of cells and biomaterials, aiming of cell proliferation and generation of innate ECM during biomaterial resorption.

Autologous TE is currently used for e.g. construction of cardiovascular or skin grafts [28, 47, 48] and breast reconstructions [49], and seems applicable for many other clinical conditions. Pre-clinical studies have shown promising results of for engineering of autologous urological grafts [50, 51], tendons [52] and constructs for the correction of CDH [53]. The progress in TE and cell-based therapies [41] makes neonatal autologous TE a realistic future alternative. This approach minimizes donor site-morbidities compared to autografts and reduces the scar sizes compared to tissue flaps (Figure 5F). In addition, side effects such as rejection or degradation and immune responses are mitigated when autologous engineered grafts are used.

Finding suitable cell sources is one of the main challenges in TE. In addition to improving the dysfunctional tissue requiring reconstruction, low immunogenicity is beneficial. To produce an autologous construct, a small number of cells can be harvested from the patient and expanded *in vitro* in specially equipped cell culture laboratories according to Good Manufacturing Practices (GMP) procedures [54]. When a sufficient number of cells have been produced, quality controls and validations are performed before the cells are combined with a biological matrix and reintroduced to the patient. The chosen cells for the constructs should have regenerative potential whilst remaining stable and not go through uncontrolled cell divisions or form teratomas. The potency of cells ranges from the immature totipotent fertilized egg, which can form all cells types in the body including the extraembryonic tissue, to the pluripotent embryonic stem cells, which can form cells from all three germ layers. The more mature multipotent stem/progenitor cells can form lineage restricted cell types e.g. hematopoietic stem cells that forms all blood cells and mesenchymal stem cells (MSC) that forms cells such as osteoblasts (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells) and myocytes (muscle cells).

1.3.1 Mesenchymal stem cells

MSC, also called mesenchymal stromal cells, are multipotent cells first described in the 1960-1970's by Friedenstein *et al.* who identified a fibroblast-like bone marrow-residing non-hematopoietic bone forming cell population [55]. These adult bone marrow MSC (aBM-MSC) showed colony forming capacity and multilineage potential when induced *in vitro*. Single-cell clones of expanded aBM-MSC retain their multilineage potential [56-58], and the single-cell colonies co-express genes characteristic for osteoblastic, chondrocytic, adipocytic, myoblastic, haematopoiesis-supporting stroma, and also endothelial, epithelial, and neuronal lineage

specific mRNA [59]. MSC are known to be immune-privileged cells; the cells escape recognition of the immune system and induce allogeneic T cell tolerance rather than cytotoxic T cell-mediated lysis [60] displaying their immunomodulatory properties. MSC possess other paracrine activities by secretion of cytokines, growth factors and microvesicles [61] and do not form teratomas or ectopic tissue after systemic administration [62]. MSC also secrete extracellular matrix molecules such as collagens, fibronectin and laminins [63] suggesting that they may play a role in the organisation of the extracellular matrix.

Besides bone marrow, MSC have further been isolated from various tissues, such as adipose tissue, skin, foetal liver, amniotic fluid (AF), placenta, umbilical cord (UC) and cord blood (UCB) but the most common source is still adult bone marrow [64-68]. Because of the diverse sources and the plethora of isolation, expansion and characterisation methods, there was a demand for a criterion to define human MSC. In 2006 the International Society for Cellular Therapy proposed the minimal criteria of MSC; 1) plastic adherence when kept in cell culture, 2) specific surface antigen expression (≥ 95 % of the population must express CD73, CD90 and CD105 measured by flow cytometry, and must lack expression of CD34, CD45, CD11b or CD14, CD19 or CD79 α and HLA class II), and 3) multipotent trilineage differentiation potential into osteogenic, adipogenic and chondrogenic lineages (Figure 6) [69].

Because of their multilineage potential, low immunogenicity, immunomodulatory characteristics and minimal oncogenic risk, MSC are clinically interesting and currently there are 938 trials involving MSC registered at Clinicaltrials.gov (2018-02-14). MSC have been tested clinically in for e.g. haematological pathologies, cardiovascular, neurological, bone, cartilage, autoimmune and inflammatory diseases and to support solid organ transplants [70]. Up to the present, thousands of patients have received treatment, mostly with adult MSC, with few adverse events reported. A meta-analysis of the randomised clinical trials was performed, and no severe adverse events was reported, although an association between MSC and transient fever was observed [71].

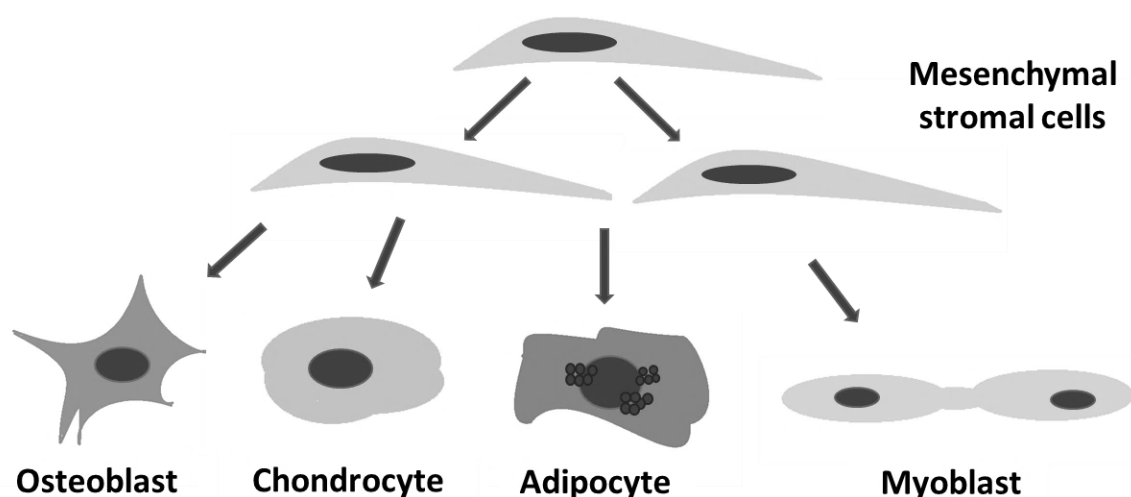


Figure 6. MSC can multiply and differentiate to mature mesenchymal cell lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts.

Cartoon made by the author.

Foetal MSC (fMSC; of 1st to 3rd trimester origin) display characteristics similar to aBM-MSC; they have the same morphological features, the cells inhibit mitogen stimulated T cell proliferation and differentiate to osteo-, adipo- and chondrogenic lineage when induced [72]. But fMSC have some advantages compared to adult MSC relevant to cellular therapies. They are found at a higher frequency, possess greater colony-forming capacity [73], express pluripotency markers (Oct-4, Nanog, Rex-1, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81), have

longer telomeres and have more proliferative potential than adult MSC and are not as lineage restricted as the adult counterpart [67, 74]. fMSC also display better engraftment than adult MSC; there was a 10-fold engraftment efficacy of fMSC compared to adult, when injected into foetal immunodeficient mice [75]. In 2005 Le Blanc *et al* could present long-term bone engraftment of foetal donor MSC after an *in-utero* transplantation of a foetus diagnosed with a brittle bone disease [76] indicating homing capacity of the fMSC besides engraftment capacity. Due to their favourable immunological, proliferative and multipotent characteristics, foetal MSC also seems to be prominent candidates for tissue engineering.

1.3.2 Potential sources for cell/tissue harvest and autologous TE

It would be beneficial if the cells used for neonatal autologous TE could be harvested during the pregnancy or at delivery, allowing generation of a construct to be used shortly after birth. The potential cell/tissue sources we suggest are therefore pregnancy-related intrauterine or term foetal or extraembryonic tissues (Figure 7). The cell source should be easy accessible and the collection not pose any considerable risk for the mother or for the foetus/new-born baby. The tissue/cell source to be used in TE and the isolation method should be robust and clinically feasible, preferable with a minimal need of handling of the cells or tissue. The cell yield should be relatively high and the retrieved cells or tissue should remain viable when combined with a matrix or supportive carrier mesh. The chosen matrix and carrier mesh should resist mechanical and tensile stress, be biodegradable and provide a satisfying environment for the seeded cells or applied tissue and not induce an immune reaction.

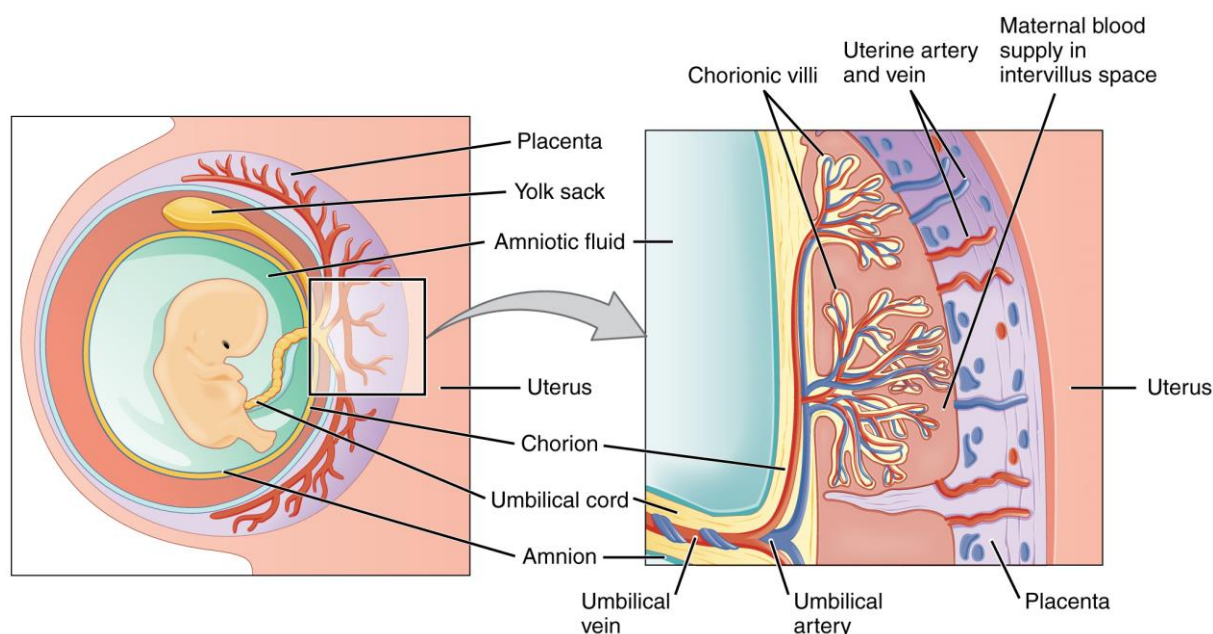


Figure 7. Illustrations of the intrauterine pregnancy-related structures, with the foetus in the amniotic sac surrounded by the protective amniotic fluid and nourished by the umbilical cord which connects the foetus with the placenta. The placenta consists of the maternal decidua, the foetal chorion membrane and the innermost foetal amnion membrane surrounding the foetus in the amniotic fluid. The blood and gas exchange occur in the intervillous space between maternal blood and the foetal chorionic villi sprouting from the umbilical cord.

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<http://cnx.org/content/col11496/1.6/>, Jun 19, 2013

1.3.2.1 Amniotic fluid

After finding a morphological anomaly in the foetus by ultrasound, the pregnant woman typically is offered an amniocentesis to exclude chromosomal abnormalities. This is a procedure where a needle is placed into the amniotic sac to collect cells that are suspended in the AF. The test is routinely performed in hospitals and is associated with a low risk of miscarriage (<1 %) [77]. At the time of amniocentesis, additional AF could be collected without extra risks for the mother or the foetus. During the last decades there have been several reports on AF-derived Oct-4 expressing progenitor and stem cells with ability to multilineage differentiation but with various degree of mesenchymal surface marker expression [78-83]. AFC also displayed therapeutic potential with the purpose of co-transplantation of AFC with UCB from the same donor to enhance engraftment of hematopoietic stem cells [84]. AF has also been investigated as a source for TE; functional human heart valves have been produced by seeding AFC onto scaffolds [85], other experimental studies suggested AFC as cellular source for bone, skin and cardiovascular TE [86-88] indicating AF as a promising source for neonatal cell therapy.

1.3.2.2 Foetal biopsies

Today, foetal biopsies are being used for prenatal diagnosis of complicated diseases, such as Duchenne and Becker muscular dystrophy, when amniocentesis or chorionic villi biopsies (CVB) do not provide trustworthy results [89, 90]. It has been demonstrated that foetuses, if wounded, do not form scar in the 1st and 2nd trimester [91, 92], which would be the time point for the amniocentesis and potential skin biopsy. Adult and neonatal skin-derived stromal cells show multilineage potential and could, besides osteo-, chondro- and adipogenic lineage also differentiate to endothelial-like cells with angiogenic properties similar to human umbilical vein endothelial cells (HUVEC) and to hepatocyte precursor cells. The cells had high proliferative capacity and expressed mesenchymal surface markers (CD73, CD90 and CD105), and not hematopoietic or endothelial surface markers (CD14, CD31, CD34, CD45 and HLA-DR) [93, 94]. Adult autologous dermal stem cells have been used for skin and hair follicle regeneration and for the treatment of diabetes-associated skin wounds [95]. The use of fetal biopsies might involve ethical considerations compared to the use of extraembryonic tissue [96], but MSC isolated from the dermis shows highly promising results as a cell source for regenerative medicine [97].

1.3.2.3 Extraembryonic tissue

The late extraembryonic tissues consist of the UC and the fetal membranes chorion and amnion of the placenta, formed from the early mesoderm, yolk sac and allantois [98, 99]. Extraembryonic tissues contains stem cells and UC and placenta are considered as waste after delivery, making isolation of fetal stem cells feasible and the tissue is readily available in fairly large quantities [100]. To avoid maternal contamination in a foetal autologous construct the amnion and the UC are the preferable extraembryonic sources when the chorion is partly fused with the maternal decidua [101]. Isolation of MSC from term UC has been extensively reported and UC seems to be a promising cell source that need further elucidation [102]. UC-MSC have been shown to be immunosuppressive, anti-inflammatory and able to escape host rejection when xeno-grafted into a mouse model [103]. The cells also show multipotency qualities; when induced, UC-MSC could, in addition to osteo-, adipo- and chondrogenic lineages, differentiate to sweat gland cells and epidermal-like cells *in vitro* [68, 104, 105]. UC-MSC can also form epithelial cells, support angiogenesis and enhance bone formation *in vivo* [106]. The amnion membrane from the placenta also contains multipotent cells, both in the epithelial and the mesenchymal layer, and possesses both anti-inflammatory and

regenerative properties and also anti-fibrotic, anti-microbial, immune suppressive and angiogenic properties [107-111]. Amnion has been used in regenerative medicine since 1910 when Davies showed superior clinical results by using amnion as skin substitute compared to xenografts [112]. The amnion is easy accessible and large pieces can be obtained immediately after birth, making it a desirable autologous tissue source for the correction of congenital soft tissue defects.

1.3.2.4 Umbilical cord blood

UCB contains several types of stem and progenitor cells and has been used for transplantation to cure malignant, metabolic, immunological disorders and other severe diseases for more than 30 years [113-115]. A large volume of UCB can be collected after birth and the mononuclear cell (MNC) fraction, including hematopoietic stem cells, MSC and endothelial progenitor cells [116], can be isolated. In pre-clinical studies, transplantation of UCB-MNC showed promising results for neonatal hypoxic–ischemic encephalopathy [117], and UCB-MNC also show angiogenic properties and supported endothelial capillary network formation *in vitro* [118]. UCB-derived endothelial progenitor cells have been used for fabrications of vascular grafts in the treatment of congenital malformations [119]. There have been reports of isolation of MSC from UCB, with various degree of success depending on time from UCB collection to MSC isolation, UCB volume obtained, culture conditions and MNC starting density [120-122] making UCB a rather troublesome source of MSC.

From UCB, plasma rich of growth factors (PRGF) can be isolated [123]. Both experimental and clinical studies have shown that PRGF is clinically safe, promotes vascularisation, tissue regeneration and wound healing [124, 125]. Plasma from UCB contains high levels of angiogenic, anti-apoptotic and immunomodulatory growth factors and various chemokines [126], and platelet-rich plasma is used clinically for treatment of many conditions such as muscular injuries [127] and bone repair [128], and seems promising in sports medicine [129].

1.4 AIM OF THE STUDIES

The aim of this thesis was to investigate potential pre- and postnatal cell and tissue sources for autologous constructs to be used for the correction of large congenital soft tissue defects.

The sources were validated in regards to accessibility, cell yield and cell properties *in vitro* such as proliferative potential, phenotype and viability, and the features of the cells and tissues when combined with a matrix or carrier mesh *in vitro* and after prolonged time in culture was investigated.

Specific aims:

1. To characterise the cell content in AF from normal pregnancies in regards to cell number, lineage commitment/identity and to investigate the cell potency as source for autologous TE.
2. To evaluate foetal subcutaneous biopsies, UC and UCB potency as cellular sources for autologous MSC to use in TE as a treatment for infants with congenital soft tissue malformations.
3. To investigate the survival of the cells on different matrices to determine the optimal combination of cells and matrix to be used in TE and transplantation.
4. To evaluate the potential of amnion from term placenta and gel manufactured from PRGF in combination with MNC both from UCB as prospective sources, combined with a supportive matrix for use in TE in the surgical treatment of infants with congenital soft tissue malformations.

2 MATERIALS AND METHODS

The methods used in the studies are described in detail in the papers included in this thesis. Some of the materials and methods below are not included in the papers. Many of these studies had, to our knowledge, not been performed previously and were attempts of developing new methods and sources for cell isolation. The studies led to increased knowledge and conclusions and are thereby included in the thesis.

2.1 ETHICS

All samples were obtained from healthy donors after informed oral and written consent according to ethical approval from the Regional Ethical Review Board in Stockholm (Dnr 2000/446/00 [study II], Dnr 2006/308-31/2 [study II] and Dnr 2012/480-31/1 [study I and III]) and in accordance with the Declaration of Helsinki. Samples with an un-normal karyotype or known malformations were excluded from the studies.

2.2 MATERIALS

2.2.1 Cell isolation and culture conditions

The isolation and culture conditions of cells and tissue in study I-III of this thesis are further described in detail in the published papers. This section presents an overview of the various isolation methods used for AFC and the different cell culture medium used to isolate and expand cell populations from AF, listed in Table 2. Additional cell culture medium in study III are also listed in Table 2.

After successful isolation of cells, the cultures were maintained at 37°C in a humidified environment containing 5% CO₂ and media was replaced every 3-4 days. At approximately 70% confluence, the cells were detached by treatment with 0.05% trypsin and 0.53 mM EDTA and replated at a density of 4000 cells per cm². Each detachment is considered as a passage (P) of the cells, e.g. first detachment and re-seeding is considered as P0-1.

Table 2. Summary of the different cell culture medium and AF cell isolation methods used in this thesis. All cell culture medium also contains 1 % antibiotic/antimycotic solutions.

Cell culture medium and isolation methods	
Plastic adherence	DMEM medium: Dulbecco's modified Eagle medium-low glucose, 10 % fetal calf serum (FCS).
	MEM-alpha medium: Minimum Essential medium alpha, 15 % FCS.
	Chang medium: MEM-alpha, 15 % FCS, 18 % Chang B, 2 % Chang C.
Coated cell culture surfaces	MEM-alpha medium: (see above)
	hESC medium: Knockout DMEM, 20 % serum replacement, 1 % Glutamax, 1 % non-essential amino acids, 0.1 mM 2-mercapto ethanol and 8 ng/mL basic fibroblast growth factor.
	Endo medium: Human Endothelial SFM medium, 10 % FCS, 20 ng/mL basic fibroblast growth factor, 1 mg/mL epidermal growth factor and 1 mg/mL fibronectin.
	DMEM-EC medium: DMEM-high glucose, 10 % FCS, 10 ng/mL epidermal growth factor, 2 mM L glutamine, 1% nonessential amino acids, 55 µM 2-mercaptoethanol and 1 mM sodium pyruvate
	Mosaic medium: BD Mosaic xeno-free culture medium, 1.6 % BD Mosaic xeno-free supplement
On feeder cell layer	MEM-alpha medium: (see above)
	hECS medium: (see above)
Positive selection	MEM-alpha medium and CD117
	MEM-alpha medium and CD133
	MEM-alpha medium and CD271
	MEM-alpha medium and Fibroblast marker
Other medium used	DMEM-F12 medium: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, 10 % FCS and 1 % L-glutamine
	RPMI medium: RPMI 1640 medium, 10% pooled inactivated human AB serum and 20 mM L-glutamine

2.2.2 Amniotic fluid (Study I)

AF was obtained at routine amniocentesis for foetal karyotyping in 2nd trimester pregnancies (n=82). The AF was centrifuged, the supernatant was removed and the AFC were resuspended in the remaining AF.

The isolation and expansion potential of AFC was evaluated with different previous published isolation methods and established culture medium:

- 1) Through plastic adherence and culture in different established MSC cell culture media containing FCS (DMEM, MEM-alpha and Chang medium, formulas in Table 2) [130-132].
- 2) With selection of CD117 (stem cell factor receptor, c-kit) [133], CD133 (prominin-1) [85], CD271 (low-affinity nerve growth factor receptor) [134] and fibroblast cells (Fibr.) [135] cultured in MEM-alpha. Both the positive (+) and the negative (-) cell fractions were analysed.

We also conducted a study aiming to improve the quality of the cells obtained from AF by:

- Following previously published protocols or by applying isolation and expansion methods used for other cell types (such as human embryonic stem cells (hESC), oral mucosa fibroblasts, epithelial, endothelial and urothelial cells) [136-140].
- By testing a new cell culture media that not yet entered the market (BD Mosaic xeno-free cell culture medium). All samples were expanded and, if successful, evaluated for proliferation, protein expression and differentiation to bone and fat cells.

2nd trimester AF was collected, centrifuged and the cell pellets retrieved were seeded accordingly:

- 1) Isolation by differential adhesion; cell culture surfaces were coated with fibronectin and the first cell fraction was allowed to adhere to the culture plastic for 30 min or 5 days. Thereafter the non-adhered cells were transferred to a new fibronectin coated cell culture well and both fractions were expanded in MEM-alpha (n=4).
- 2) The cell pellet from the AF were seeded onto collagen IV coated culture surfaces and expanded in hESC media (formula in Table 2, n=4).
- 3) The cell pellet from AF were seeded onto 1 % gelatine coated culture surfaces and expanded in epithelial cell culture medium (DMEM-EC n=4) or in endothelial cell culture medium (Endo n=4) (formulas in Table 2).
- 4) Human aBM-MSC was treated with Mitomycin C to inhibit cell proliferation and seeded 24 h in advance as feeder cells for the isolation process. The AF cell pellet were seeded onto the feeder cell layer and expanded in MEM-alpha (n=3) or hESC media (n=3).
- 5) AF obtained were split into two equal fractions and cells were seeded onto either collagen (n=2) or fibronectin (n=4) and expanded in BD Mosaic xeno-free culture media (formula in Table 2) or MEM-alpha for comparison.

2.2.3 Foetal subcutaneous cells (Study II)

In order to simulate how 2nd trimester foetal biopsies could be obtained in clinical situations subcutaneous biopsies were collected with punch biopsy needles from elective terminations at gestational week (GW) 20-21 (n=2). The tissue pieces were placed in cell culture dishes, left to adhere to the plastic for 10 min before addition of the cell culture medium, allowing foetal subcutaneous cells (fSC) to migrate out from the tissue to the surrounding culture surface

(explant method). After 7 days in culture the tissue pieces were removed and the retrieved fSC was expanded in MEM-alpha culture medium.

2.2.4 Placenta

2.2.4.1 Chorionic villus biopsies

CVB were obtained from routine sampling for foetal karyotyping at GW 11-14 (n=11) with an 18-gauge needle (1 mm ϕ). Tissue pieces, approximately 1-3 mm long, were placed into cell culture dishes and cells were isolated with above mentioned explant method and thereafter expanded in BD Mosaic xeno-free culture medium or MEM-alpha.

2.2.4.2 Amnion membrane (Study III)

The foetal amnion membrane was mechanically isolated from term placentas retrieved after elective caesarean sections (n=4). The amnion pieces were maintained in MEM-alpha culture medium until further analysis or included in constructs (see below).

2.2.5 Umbilical cord

UC was collected after elective caesarean sections of term deliveries. Cells were isolated from the UC (n=2) by 3 different methods:

- 1) Explant method with 1x1 cm pieces of the connective tissue (Wharton's jelly) from the UC, as described above.
- 2) Enzymatic dissociation (collagenase, hyaluronidase and trypsin) of UC Wharton's jelly pieces to single cells and seeded in culture flasks.
- 3) Mechanical dissection of arteries from the UC and enzymatically treated as described above.

All UC samples from the different isolation methods were cultured in DMEM and MEM-alpha culture media for comparison.

2.2.6 Umbilical cord blood

Fresh UCB units were collected from term deliveries by the altruistic Swedish National Umbilical Cord Blood bank at Karolinska University Hospital. When the cell count was too low for clinical banking, the fresh UCB could be donated to research. The UCB was collected before delivery of the placenta into a UCB collection bag containing 21 mL anticoagulant (citrate phosphate dextrose solution).

2.2.6.1 UCB-MSc

To isolate MSc from UCB (n=11), the MNC fraction was isolated by density gradient separation (Lymphoprep, density 1.077 g/mL). The MNC were seeded in cell culture flasks at different cell densities, with or without addition of dexamethasone to the DMEM cell culture medium.

2.2.6.2 MNC enriched PRGF gel (Study III)

To obtain a MNC-enriched gel of PRGF, UCB collected as described above were divided into 2 parts (n=3): One part for isolation of MNC (described above) and one part for isolation of PRGF. For isolation of PRGF, the UCB was separated into red blood cells at the bottom of the tube, PRGF in the middle layer, and plasma poor in growth factors (PPGF) in the top layer by centrifugation. The PPGF layer was discarded, the remaining PRGF was transferred to a new

tube and mixed with the isolated MNC, and thereafter 10 % $\text{CaCl}_2 \text{H}_2\text{O}$ was added for gel formation (Figure 8A-D).

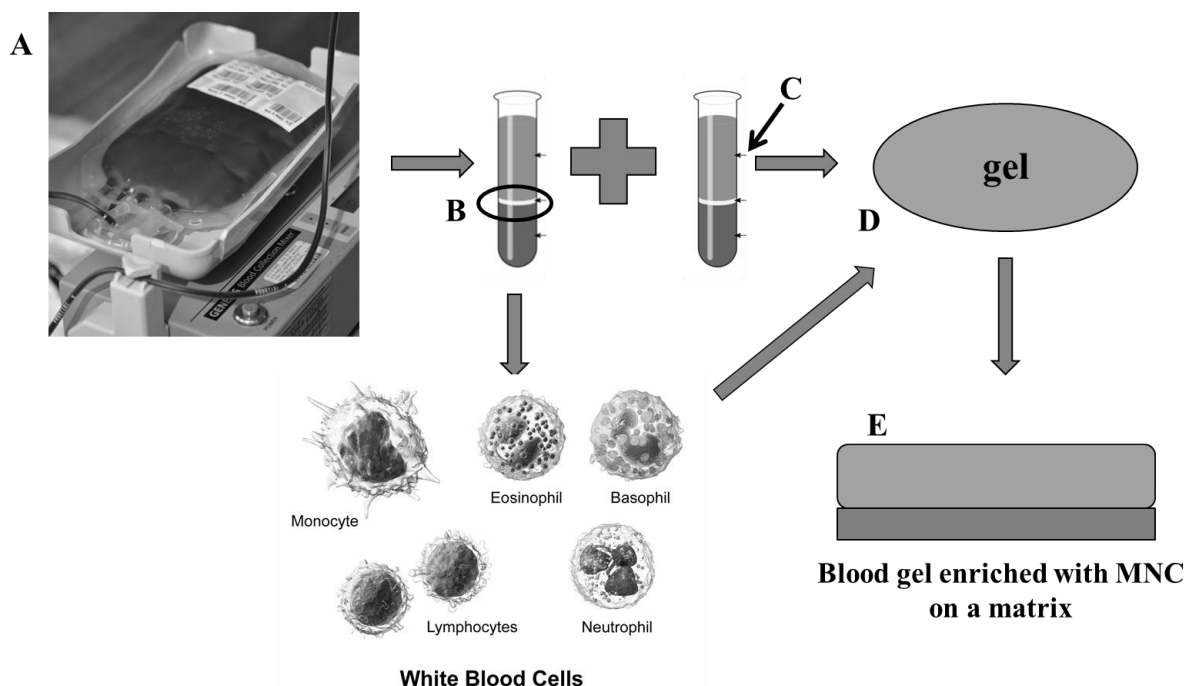


Figure 8. Production of MNC enriched PRGF gel and PRGF-SIS constructs. **A)** UCB units containing anticoagulant were divided in 2 parts; **B)** for MNC isolation and **C)** for PRGF separation. **D)** The MNC and PRGF is mixed and **E)** gel is cast on top of SIS to form the construct.

Illustration produced with pictures with permission from Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". WikiJournal of Medicine 1 (2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436. KnuteKnudsen at English Wikipedia and U.S. Air Force photo by Karen Abeyasekere/Released

2.3 METHODS

The experimental methods used in this thesis are mostly based on *in vitro* models with culture-expanded cells. Using primary cells in culture with a defined culture medium is *per se* a selection of cell subpopulations and keeping cells in culture with repeated enzymatic detachment and expansion might change the cell properties compared to fresh un-cultured cells. Expansion of the cells is most often necessary to obtain satisfactory number of cells to be able to perform the experiments, and most established published methods are based on culture expanded cells.

2.3.1 Cell content in fresh AF (Study I)

To identify the cell composition of AF, freshly harvested AF was analysed for different cell lineages with 2 methods; flow cytometry and immunocytochemistry.

2.3.1.1 Flow cytometry

Fresh AF was centrifuged, the supernatant removed, the cell pellet resuspended and viable cell in the AF was counted. Because of low cell content in the AF, two to three samples were pooled before flow cytometry analysis. The cells in the AF were stained with monoclonal conjugated antibodies against CD31 (PECAM-1), CD45 (leukocyte common antigen), CD235

(glycophorin A), CD73 (5'-nucleotidase), CD117, and CD271 and acquired in a flow cytometer.

2.3.1.2 Immunocytochemistry

For immunocytochemistry analysis the AF was centrifuged, the supernatant removed and the cell pellet resuspended and spun onto glass slides and let to dry overnight. The cells were fixed with paraformaldehyde (PFA) before staining with monoclonal conjugated antibodies against CD31, CD45, and CD73. The glass slides were mounted with DAPI and examined in a microscope. Positive and negative cells were counted in three random view fields per slide.

2.3.2 Expansion and proliferation assays of isolated cells (Study I-II)

Proliferation assays were performed on all successfully isolated and expanded AFC, fSC and CVB cells.

The proliferation potential was evaluated by consecutive culturing of the cells. We also wanted to investigate when the cells would reach the *Hayflick limit* [141] and cease to divide, and kept them in culture for up to 15 P or until the cells reached senescence.

The number of population doublings (PD) was calculated using the equation:

$$PD = \log_2(y/x)$$

The population doubling time (PDT) was calculated using the equation:

$$PDT = \frac{t}{\log_2(y/x)}$$

Accumulated number of cells per cm² culture surface was calculated using the equation:

$$\frac{(y - x)}{cm^2}$$

Where y = number of cells when counted, x = initial number of cells and t = time (h) from plating to counting the cells.

The cells were stained for beta-galactosidase as a marker for senescence at P6, P10 and if possible P15. Cells positive and negative for beta-galactosidase staining were counted in five random view fields per well at 10x magnification.

The correlation between AF volume and cells retrieved at P0-1 was also analysed.

One prominent feature of MSC is the capability to form colonies at a low seeding density. To investigate the capacity of the expanded cells we performed the Colony forming units-fibroblast (CFU-F) assay. Cells were seeded at low densities in triplicates at P3 by plating 4 cells/cm² under regular culture conditions for 9 and 14 days. Colonies consisting of more than 50 cells were counted after fixation with 100% methanol and staining the cytoplasm with eosin.

2.3.3 Characterisation of culture expanded cells (Study I-III)

The characterisation assays were performed, if possible, on culture expanded AFC, fSC, CVB and UC cells. The cell morphology was examined in a bright field microscope and captured at 10-20x magnification

2.3.3.1 Phenotype of culture expanded cells

For characterisation of MSC there are yet no specific MSC surface epitopes, but according to The International Society for Cellular Therapy position statement; *Minimal criteria for defining multipotent mesenchymal stromal cells* (2006) [69], there are specific surface epitope

expressions that should be fulfilled. From these guidelines we designed a panel of antibodies for the assay. Surface epitope expression by cultured cells was analysed with flow cytometry at P3-5. The cells were stained with monoclonal conjugated antibodies against hematopoietic markers: CD14 (lipopolysaccharide-binding protein), CD34 (hematopoietic progenitor cell antigen), CD45, CD80 (B7-1), CD31, CD73, CD90 (Thy-1), CD44 (H-CAM), CD105 (endoglin), HLA class I and HLA class II, and analysed in a flow cytometer.

2.3.3.2 Differentiation assay

The ability to differentiate to mesodermal lineages *in vitro* is a hallmark property of MSC. Therefore, the differentiation assay is of great importance for the characterisation of the putative MSC. The capacity of the cells to differentiate into adipogenic and osteogenic lineages was assayed at P3-5 and differentiation was visualised by staining of lineage specific molecules.

Adipogenic differentiation was induced by addition of dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine and insulin to the culture medium DMEM-high glucose. At the end of the experiments the cells were stained with Oil Red O solution for visualisation of lipid vacuoles.

Osteogenic differentiation was induced by addition of dexamethasone, ascorbic acid-2-phosphate and β -glycerophosphate to MEM-alpha. Staining for presence of mineralisation (calcium deposits) was performed using Alizarin Red S solution.

Positive cells were analysed in a bright field microscope and captured in 10x magnification (osteogenic) or 40x magnification (adipogenic differentiation).

2.3.4 Mixed lymphocyte culture (study I and UC cells)

Mixed lymphocyte culture (MLC) is an *in vitro* model to investigate recipient immune responses induced by donor cells. To investigate whether AFC and UC cells induced an immune response, adult MNC were co-cultured with 1, 10 and 50 % irradiated AFC/UC cells in triplicate and incubated for 6 days. On the fifth day, ^3H -thymidine was added to the cultures for incorporation in DNA synthesis. After 24 h the cells were harvested and radioactivity was determined as counts per minute (cpm). The percent proliferation was calculated using the following equation:

$$\text{Percent proliferation} = \frac{\text{cpm PBL} + \text{AFC/UC cells}}{\text{cpm PBL}} \times 100$$

2.3.5 Angiogenic properties of fSC (study II)

Angiogenesis is crucial for graft survival and the clinical outcome. To investigate the angiogenic properties of fSC, HUVEC were seeded into basement membrane extract pre-coated wells. As culture medium either regular endothelial cell (EC) medium or medium with a ratio of 1:1 EC:conditioned medium (CM) from fSC culture (CM-fSC) or 1:1 EC:fSC regular culture medium (MEM-alpha) was used. After 16-18 h incubation the tubes formed by HUVECs were visualized using an inverted bright field microscope, images of the wells were taken at 4x magnification and tube formation quantified (Nb meshes) with the software ImageJ (National Institutes of Health, USA).

2.3.6 fSC/matrix constructs (study II)

2.3.6.1 Cell proliferation in collagen gel

To investigate if the collagen gel provides a favourable milieu for the fSC to remain viable, a proliferation assay in the collagen gel was performed. fSC were stained with carboxyfluorescein succinimidyl ester (CFSE) and resuspended in MEM-alpha. Collagen gels were prepared according to the manufacturer's protocol and casted in 12-well plates. Thereafter 4000 cells/cm² were seeded on top allowing the cells to migrate into the gel and the gels containing the cells were kept in culture. To measure the cell proliferation, the collagen gels with cells were digested with collagenase I and trypsin. The cell proliferation was analysed by flow cytometry at day 0 (before seeding onto the gel), 3, 7, 10 and 14.

2.3.6.2 Construction of fSC in collagen with PCL

To evaluate the cell properties in the collagen gel when combined with poly(ϵ -caprolactone) (PCL), cell/collagen-PCL constructs were analysed. The construct consisted of two outer layers of collagen type I gel mixed with cells and with an inner core of 0.25 mm PCL knitting, kindly provided by Ajalloueian *et al.* [142] (Figure 9). The PCL mesh structure had been prepared with 35 needles per inch with a needle gauge of 75 to produce a porous monofilament structure. To enhance the mechanical strength, excess fluid was removed by the method plastic compression, forming a thin hybrid scaffold. The constructs were cut into pieces and were maintained in culture. After 1, 2, 3, 6 and 8 weeks, the constructs were collected and fixed in PFA, paraffin embedded, sectioned and analysed as described below.

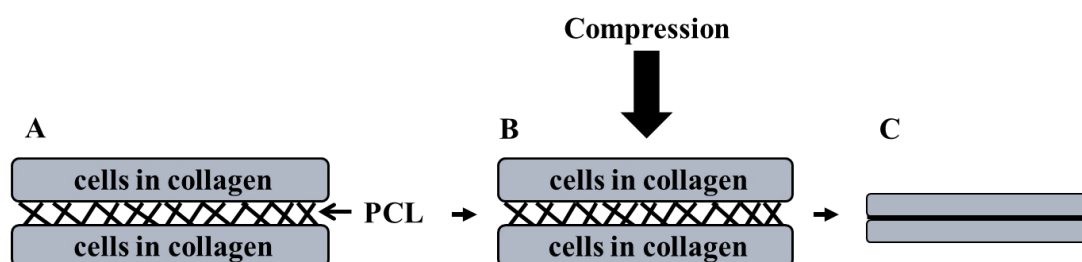


Figure 9. Schematic illustration of the production of the cell/collagen-PCL construct. **A)** Collagen gels with cells were cast with PCL as a supportive core. **B)** Excess fluid is compressed out from the construct forming **C)** a thin hybrid scaffold with enhanced mechanical strength. Illustrations by the author.

2.3.6.3 Construction of fSC with SIS

To investigate cell integration and cell properties when seeded onto 4-layer SIS (Biodesign or Surgisis, Cook Biotech Incorporated), fSC-SIS constructs were produced and analysed. SIS was rehydrated and placed in an ultra-low attachment plate and fSC were added on top. After 1 and 2 weeks in culture, the constructs were fixed with PFA, paraffin embedded, sectioned and analysed as described below.

2.3.6.4 Visualisation of cells in matrices

To visualise and localise the cells in the constructs, the sections were stained with haematoxylin (HTX) and eosin. To investigate cell proliferation, sections were stained with Ki67. Sections were also stained for α -smooth muscle actin (α -SMA, cytoskeleton), pan-cytokeratin (PCK, epithelium), CD31 (endothelium) and CD73 (common stromal/mesenchymal marker) and counterstained with HTX.

2.3.7 Placenta (study III)

2.3.7.1 Comparison of culture conditions of amnion

Amnion consists of an outer epithelial cell layer and an inner MSC-containing connective tissue layer. To optimise culture conditions, three different culture media were initially compared in respect to amnion-residing cell morphology, viability, and proliferation. The culture media compared were (see formulas in Table 2):

- 1) Mesenchymal stromal cell culture medium (MEM-alpha)
- 2) Epithelial cell culture medium (DMEM-EC)
- 3) Endothelial cell culture medium (DMEM-F12)

Pieces of amnion were equally divided between the different culture conditions and kept in culture. At days 0, 3, 7, 10, 14, and 21, the amnion pieces were fixed in PFA, paraffin embedded, sectioned, and stained with HTX/eosin and Ki67 as described above but also for activated caspase 3 as a sign for apoptosis.

2.3.7.2 Characterisation of migrated amnion-derived cells in the different culture conditions

When the amnion was kept in culture, mono-layers of adherent cells were noted in the cell culture dishes. We investigated whether the cells were of epithelial or mesenchymal origin and if the culture conditions induced any differences. To characterize the amnion derived cells (ADC) that had adhered to the cell culture surface, the cells were harvested at day 21, stained with monoclonal antibodies against CD31, CD73 and PCK and thereafter analysed in a flow cytometer.

2.3.7.3 Characterisation of Amnion-Residing Cells in Constructs with SIS

To analyse if the properties of the amnion-residing cells would be altered when combined with SIS (amnion-SIS), pieces of amnion were placed on dry 4-layer SIS and let to adhere for 5 minutes. To increase the contact, the amnion was sutured onto the SIS matrix and the constructs were kept in culture. At days 0, 3, 7, 10, 14, and 21 amnion-SIS pieces were transferred to PFA for fixation and thereafter paraffin embedded and sectioned. The sections were stained with HTX/eosin, PCK, CD31, CD73, Ki67 and activated caspase 3 as above. Stained sections of amnion without SIS served as a control.

2.3.8 MNC enriched PRGF gel from UCB (study III)

To evaluate the durability of the produced gel and to analyse the protein expression of the MNC in the PRGF, pieces of SIS was placed in 8-chamber slides and MNC enriched PRGF was added on top for gel formation (Figure 8E). PRGF without SIS was produced as a control. The gel pieces and the PRGF-SIS constructs were transferred to cell culture plates and RPMI 1640 medium was added. At day 0, 3, 7, 10 and 14 the PRGF gel and PRGF-SIS constructs were transferred to PFA and thereafter paraffin embedded and sectioned. The sections were stained as described above with HTX/eosin for morphology, CD34 and CD45 for surface epitope expression, Ki67 and for activated caspase 3.

3 RESULTS

3.1 AMNIOTIC FLUID (STUDY I)

3.1.1 Cell content in fresh AF

Our study showed large donor variation between the AF samples. Cell count was 1750-4750 cells/mL AF and consisted mostly of hematopoietic cells. Less than 0.5 % of the cells expressed MSC markers (CD73+/CD117+/CD271+) in the flow cytometry assay and less than 0.005 % in the immunocytochemistry analysis (CD73+). Because of the limited cell number, we could not include markers for other surface epitopes to retrieve a more detailed analysis.

3.1.2 Expansion and proliferation assays of isolated AFC

Isolation of AFC was successful for all samples except for CD117+, CD271+ and CD271- cells. Due to the variable isolation success, some experiments could not be carried out in all AFC isolations, since not enough cells could be expanded.

The expansion potential of AFC was analysed by investigating multiple parameters. The time in P0 (before trypsination) did not differ but the number of cells obtained per ml AF in P0-1 varied; cells expanded in Chang medium gave the highest yield and CD271+ isolated cells the lowest. CD117+ isolated AFC had the highest cumulative number of cells at P5 and CD271+ AFC the lowest. AF volume did not correlate with the number of cells obtained per ml ($r = -.4$). Overall, CD117+ AFC showed the best proliferative capacity with highest cumulative number of cells, with most PD and the shortest PDT in P1-5. In the CFU-F assay the CD117+ samples were 100 % confluent compared to CD271+ cells with 4 colonies per 120 seeded cells after 14 days in culture. The lowest fraction of senescent cells in P10 was also found in CD117+ AFC, but not all samples could be analysed in P10 due to the cell's incapability to proliferate.

None of the other investigated isolation and expansion methods (section 2.2.2) showed any tendencies to improve the quality of the cells, rather the opposite (data not shown).

3.1.3 Characterisation of culture expanded AFC

Generally, the AFC cultures consisted of heterogeneous cell populations with a predominance of fibroblastic cells but there were also epithelial-like cells in the early passages.

3.1.3.1 Phenotype of AFC

The phenotype of AFC cultured for 3-5 P was analysed with flow cytometry. All AFC were negative for CD14, CD31, CD45, CD80 and HLA class II and expressed CD44, CD73, CD90, CD105 and HLA class I, although at different levels. It is notable that cells cultured in Chang medium had low expression of CD90, CD133+ cells had high expression and the other AFC had intermediate expression. Furthermore, CD105 was expressed at a low percentage by all cells except cells cultured in DMEM that had intermediate expression. Cells cultured in DMEM had intermediate expression of CD34 and CD133+ or CD271- cells had low expression.

3.1.3.2 Differentiation ability of culture expanded AFC

The ability of AFC to differentiate into osteogenic and adipogenic lineages was analysed and the results showed great variation and a general low success. The experiments were repeated several times and control cells (aBM-MSC) exhibited positive differentiation. All cells cultured

in MEM-alpha, CD117+/-, CD271 +/- and Fibr- cells differentiated into the osteogenic lineage and 40-66 % of the of CD133+/-, Fibr+ and DMEM samples. None of the cells cultured in Chang differentiated into the osteogenic lineage. Only CD271- cells showed 100 % differentiation into the adipogenic lineage. Between 33-50 % of the CD117-, CD133+, Fibr- and DMEM cells differentiated into adipogenic cells. None of Chang, MEM-alpha, CD117+, CD133-, CD271+ or Fibr+ cells differentiated into the adipogenic lineage.

3.1.4 Mixed lymphocyte culture, AFC

To analyse if AFC induce an immune response, the cells were co-cultured with allogeneic MNC and the percentage of proliferation compared to the negative control was calculated. AFC cultured in DMEM induced the highest, but still a weak immune response with a four-fold increase in MNC proliferation, equated with the positive control (MNC from a mix of 6 allogeneic donors) where the proliferation increased 20 times compared to negative control (MNC without any allogeneic cells).

3.2 FOETAL SUBCUTANEOUS BIOPSIES (STUDY II)

3.2.1 Expansion and proliferation assays of isolated fSC

Cells could be isolated from 2nd trimester subcutaneous tissues and expanded in cell culture. The cells could be kept in culture for up to 13 P before they became senescence, with a total number of 43 ± 3 PD and an average PDT of 39 ± 8 h at P1-6. After P7 the PDT increased with every passage. At P6, 3 % of the fSC were positive for beta-galactosidase in the senescence staining and at P10, 10 % were positive.

The cell colony forming capacity was analysed using the CFU-F assay. At day 9 fSC had formed 14 ± 11 colonies consisting of more than 50 cells. UC-MSc formed 2 ± 1 and aBM-MSc 2 ± 2 colonies (mean \pm stdev) under the same conditions. At day 14 the fSC were 100 % confluent and colonies could not be counted.

3.2.2 Characterisation of culture expanded fSC

All cell cultures consisted of homogeneous fibroblast-like cell populations, visualised by bright field microscope and captured at 10x magnification.

3.2.2.1 Phenotype of cultured fSC

As analysed by flow cytometry, the cells expressed CD44, CD73, CD90 and HLA class I but not CD14, CD31, CD34, CD45, CD80 or HLA class II on the cell surface. The expression of CD105 was intermediate (range 63-67 % positive cells).

3.2.2.2 Differentiation ability of fSC

When induced, fSC readily differentiate into both adipogenic and osteogenic lineages *in vitro* in less than 21 days, as demonstrated by positive staining for Alizarin Red S and Oil Red O.

3.2.3 Angiogenic properties of fSC

Angiogenic properties by fSC were analysed in a tube forming assay. There were no significant differences in tubes formed with or without fSC; HUVECs in EC medium formed 29 ± 13 tubes, HUVECs in 1:1 EC:fSC-CM formed 34 ± 7 tubes and HUVECs in of 1:1 EC:MEM-alpha formed 34 ± 4 tubes (n = 6 per group, Figure 10). All values are presented as mean \pm stdev.

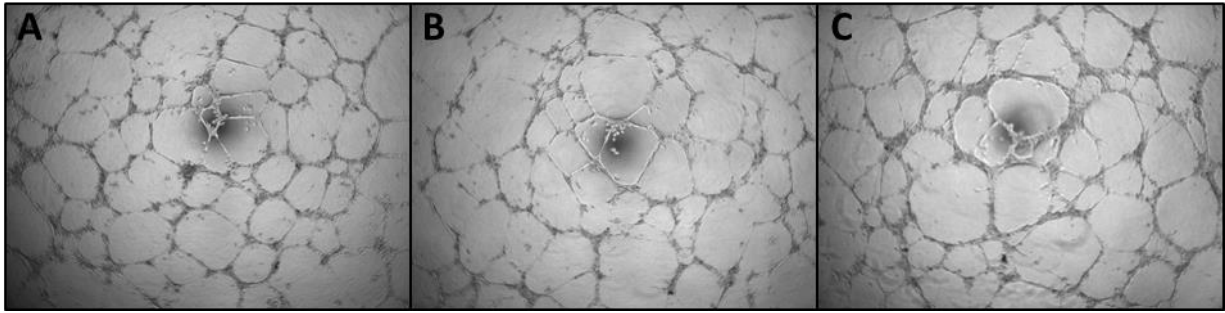


Figure 10. The angiogenic properties of fSC were investigated. There were no significant differences between the different culture conditions. Representative pictures of tubes formed in **A)** EC medium, **B)** 1:1 EC:MEM-alpha and **C)** 1:1 EC:fSC-CM. Magnification 4x. Photos by the author.

3.2.4 fSC/matrix constructs

3.2.4.1 fSC proliferation in collagen gel

The capacity of fSC to proliferate in the collagen gel was measured using CFSE staining. The cells proliferated and migrated into the gel until the endpoint at 14 days. The proliferation index increased 2.3 ± 1.2 times (mean \pm SD) up to day 10 compared to day 0, thereafter the proliferation curve plateaued.

3.2.4.2 Construction of fSC and SIS or in collagen with PCL

fSC were seeded onto rehydrated SIS or in collagen-PCL constructs. After 1 and 2 weeks the cell-matrix constructs were analysed. The cells on SIS covered the surfaces but had not integrated deep into the matrices as opposed to the collagen constructs where cells were distributed throughout the gel in addition to lining the gel surface.

All cells expressed CD73 and there were Ki67 positive cells at all time points in both matrices. fSC on SIS had lower expression of α -SMA compared to fSC in collagen-PCL. There was no expression of CD31 or PCK in any of the constructs.

To investigate if the cells in the collagen-PCL constructs would turn into more endothelial- or epithelial-like cells, the cells in the constructs were kept in culture for another 3, 6 and 8 weeks and stained for the above-mentioned markers. The HTX/eosin staining showed increasing numbers of cells, and thereby an increased expression of CD73 and α -SMA, but no expression of CD31 or PCK could be detected. At week 8 the top layers of collagen had high cell density due to cell proliferation and were partly detached from the PCL mesh and could not be properly analysed.

3.3 PLACENTA

3.3.1 CVB samples

Cells could be expanded from five of 11 CVB-samples, whereof one in BD Mosaic medium. No cells showed proliferative capacity and therefore no samples were further investigated.

3.3.2 Amnion (Study III)

3.3.2.1 Comparison of culture conditions of amnion

The amnion could easily be isolated from the placentas and kept in culture to the endpoint of 21 days. To evaluate different culture medium, amnion pieces were cultured in MEM-alpha, DMEM-EC and DMEM-F12. The amnion pieces were evaluated morphologically with HTX/eosin, which showed that the mesenchymal cells exhibited maintained morphology throughout the time in culture but there was a decreasing integrity of the epithelial layers at the end of culture in all conditions. There were no differences in proliferation and viability of the amnion-residing cells when testing the different culture medium. A uniform low expression of Ki67 was detected at all time points up to day 21 when no expression was detected. Expression of caspase 3 was low, but slightly increased over time.

3.3.2.2 Characterisation of migrated ADC in the different culture conditions.

The ADC in MEM-alpha and DMEM-EC showed fibroblast-like morphology whereas the cells in DMEM-F12 had neither typical epithelial nor fibroblast like morphology. The ADC were further investigated by surface epitope expression by flow cytometry, and the majority of cells from the different culture conditions expressed CD73 with a significantly higher expression in DMEM-EC compared to the other cell culture media (DMEM-F12 66.7 %, MEM α 87.8 % and DMEM-EC 96.6 %, $p = 0.03$ and 0.008 respectively). There was no expression of CD31 or PCK of the ADC in any of the culture conditions. Based on these results, DMEM-EC was selected for the remaining experiments.

3.3.2.3 Characterisation of Amnion-Residing Cells in Constructs with SIS.

To investigate whether the amnion-residing cells would migrate and integrate into the SIS and to analyse protein expression, amnion pieces were combined with SIS and kept in culture for 0-21 days and thereafter stained for protein expression. Amnion without SIS served as control. The HTX/eosin staining showed no morphological differences compared to amnion pieces without SIS and no cells were detected inside the SIS. Cells in both the epithelial and mesenchymal layers expressed CD73 and the epithelial cells expressed PCK. The expression of Ki67, marking proliferative cells, was less than 3% at all time points up to day 21 where no Ki67 stained cells were detected in any samples. Overall there was a low expression of caspase 3 ($< 2\%$) in both mesenchymal and epithelial cells, and it slightly increased in the epithelial cells over time. There was no CD31 expression at any time points and there were no differences in protein expression between amnion-SIS and amnion without SIS.

3.4 UMBILICAL CORD

Cell isolation from the first UC was successful except for the enzymatic digestion of the arteries. After improvement of the method, by reducing the enzymatic treatment, cells could be isolated from the second UC with all three methods. The expanded UC cells had homogenous fibroblast-like morphology and could be kept in culture for up to the endpoint at P10. The PDT during P1-5 was 42.3 ± 7.5 h and 52.7 ± 18.7 h in P6-10. Cells from UC expressed MSC (CD44, CD73, CD90 and CD105) but not hematopoietic or endothelial surface markers (CD14, CD31, CD34, CD45, CD80 and HLA-II), but showed a low ability to differentiate into adipogenic and osteogenic lineages. UC cells derived from arteries, both cultured in DMEM and in MEM-alpha differentiated to adipogenic cells. Cells derived from tissue pieces, cultured in MEM-alpha differentiated also to adipogenic cells. No UC cells from the different treatment differentiated to osteogenic cells. UC cells showed immune suppressive characteristics with a

reduction of MNC proliferation, to a higher degree in MEM-alpha than in DMEM (45.6 % vs 8.3 %).

3.5 UMBILICAL CORD BLOOD

3.5.1 Isolation of UCB-MSC

After isolation and seeding the UCB MNC, few non-proliferating adhered cells were observed in some samples, which may have been monocytes. No fibroblast-like cells could be isolated with any of the previously published methods.

3.5.2 MNC enriched PRGF gel from UCB (study III)

The PRGF gel with or without SIS could be kept in culture for up to 14 days with sustained morphology, but with decreasing number of cells and with an increasing degradation of the gel. At day 14 the gel was completely dissolved and only SIS remained. Therefore day 14 was excluded from further analysis. There were no differences in protein expression if the PRGF gel was combined with SIS or not. The cells expressed CD45 and a few CD34 positive cells could be detected at all time points. Cells were positive for Ki67, but with decreasing number over time and at day 10 no proliferative cells were detected. The caspase 3 expression was low but increased over time.

4 DISCUSSION

The purpose of this thesis was to find a better solution to the current treatment of severe congenital soft tissue malformations. In these studies, we aimed for identifying a potent cell or tissue source for autologous constructs to be used for the correction of large soft tissue defects in new-born babies. Surgical corrections may be needed early in life; ranging from within a few hours to weeks after birth, depending on severity of the defect and clinical status of the new-born. In order to present an autologous graft ready to use for the correction we sought for cellular/tissue sources accessible during the pregnancy or at birth. We also aimed for a feasible isolation method, which would not include excessive handling or treatment of the cells and aiming to avoid or at least minimise, donor site morbidity.

In the future, the entire process from isolation of cells to construction of the autologous graft must be practical in a clinical situation. The cell processing would be performed in a GMP facility, with all substrates involved approved for clinical use by the National and International Medical Product Agencies, which is also needed to be taken into account when designing the studies. Additionally, the matrix or carrier material used for the constructs should provide a favourable milieu for the cells.

We decided to use SIS, a form of decellularized ECM, mostly collagen, which use is well established in surgical practice, and a gel formed of collagen, which is the most abundant ECM protein in the human body. Collagen alone does not provide the mechanical or tensile strength needed for a tissue graft, therefore we used PCL as a carrier matrix and plastic compression to remove excess fluid from the gel to further increase the tensile strength.

As cellular or tissue sources we decided to focus on extraembryonic tissue which is mostly regarded as waste after birth, AF and small subcutaneous biopsies which can easily be collected at amniocentesis.

4.1 AMNIOTIC FLUID (STUDY I)

In our first study we investigated AF as cellular source for autologous TE. Amniocentesis is a safe (less than 1% risk for foetal loss), sterile and quick procedure [77, 143]. An advantage is that pregnant women carrying an affected foetus are offered the procedure anyways, and most parents accept it, to exclude chromosomal abnormalities. An extra aliquot of AF can be collected without any additional interventions. Our study showed that there is high donor variability among AF and for this reason we included 82 samples and explored several isolation procedures, but still the variation was high. We could neither correlate AF volume or isolation method with a more successful outcome and the cell populations isolated and expanded from the AF were heterogeneous in many aspects. Even within the studied groups where same isolation and expansion protocols were used, some cultures became senescent at early passages (1 to 3) whereas some continued proliferating for long periods (up to 15 passages). These findings have also been reported by others groups; Roubelakis *et al.* investigated the heterogeneity of AF-derived cells (90 samples) where only a small sub-population of the AFC (spindle-shaped, SS-AFC) displayed the mesenchymal features such as proliferative and trilineage capacity, and also that SS-AFC could only be detected in 6 % of the AFC cultures after 2 -3 passages [144]. Gucciardo *et al.* analysed different expansion protocols for AFC and included >160 samples [145]. The isolation success was low and they could not maintain many cultures for more than 2 passages, all in accordance with both Roubelakis' and our findings. In freshly harvested AF we identified distinct cell populations by flow cytometry. The different populations of non-hematopoietic, non-endothelial cells were less than 1 %, and this was confirmed with immunocytochemistry. This shows that stromal cells are rare in second trimester AF, which is in line with other reported results [80, 146]. Our study shows that among

the AFC populations studied, CD117+ AFC displayed highest proliferation potential, which is in accordance with previous published results [80, 147, 148]. However, of note is that only half of our CD117+ cultures were successful, making CD117 selection of AFC an uncertain cell isolation method for our purpose.

The culture expanded AFC showed some mesenchymal features, partly by expression of typical MSC markers CD44, CD73 and CD90, however at various degrees. Several studies have shown large variations in surface epitope expression of AFC, which has been attributed to many different features like cell shape, gestational age and cell growth conditions [144, 146, 149, 150]. The herein used differentiation assays are well established in our laboratory and have been proven successful in other studies [72, 76, 151, 152], but were consequently not successful with AFC. It appeared that AFC, like other foetal cells [72, 153, 154], had a preference for the osteogenic lineage, demonstrated by the higher osteogenic compared to adipogenic differentiation in our study, in alignment with Gucciardo's published study on AFC [145]. All taken together, this study shows that AFC is a heterogeneous population of cells with uncertain isolation success and wide proliferation potential and cannot be considered as true MSC, specifically due to the low differentiation potential and divergent surface marker expression. Although of note is that the published criterion for MSC was created for adult cells derived from bone marrow [69].

To conclude study I, we could not see a positive trend for isolation and expansion of AFC with any method; the samples that persisted in culture did so because of cells present in the starting material rather than because of the isolation method or culture condition. In a clinical situation, AF is too unreliable and donor-dependent source for cell harvesting and use in autologous TE.

4.2 FOETAL SUBCUTANEOUS BIOPSIES (STUDY II)

After these rather unsatisfying attempts to isolate and expand perinatal cells from several sources, we proceeded to another prenatal cell source; 2nd trimester subcutaneous biopsies. It might seem as a quite invasive and aggressive approach to retrieve cells for an autologous construct but compared to postnatal harvest of sufficient number of cells, the donor site area with subsequent morbidity and scar formation is hugely diminished by performing the harvest prenatally. The postnatal by default healing pathway after injury or invasive procedures is scar formation. Collagen in the ECM is exposed at injury and activates platelets, which induce a cascade of events including inflammation and fibroblast recruitment for tissue restoration. The fibroblasts produce collagen which in the end leads to disorganized ECM collagen fibres and different degrees of scar formation [155, 156]. However, the foetal wound healing pathway differ somewhat to the postnatal; foetal platelets are not as reactive to collagen leading to reduced inflammation and less collagen production, resulting in tissue regeneration without scarring, instead of tissue restoration with scars [92, 157].

It is believed that fetuses can experience pain already at 20 weeks of gestation and they therefore receive analgesia before intrauterine interventions such as fetoscopy, infusions or biopsies either direct or indirect by placental transferred drugs administered to the mother. The foetus is also sedated in order to immobilise the foetus for a safe procedure and uterine relaxation to reduce the risk of preterm labour [158, 159]. Our study was a proof of principle; we had only two donors from GW 20-21. The access to non-chromosomal aberrant donors in late gestation is limited, but in a clinical situation that would be the time point for tissue collection after the diagnosis of a soft tissue defect. The study showed that isolation and expansion of fSC from a small biopsy is feasible and the isolated cells possessed a high proliferative capacity and went through up to 45 PD before they ceased to grow, all in accordance with the *Hayflick limit*, indicating that the fSC had a normal, non-transformed cell phenotype [141]. The culture-expanded fSC exhibited high expression of the MSC markers and

readily differentiated to osteogenic and adipogenic lineages. The surface marker expression and the differentiation capacity indicated mesenchymal features of fSC according to the International Society for Cellular Therapy minimal criteria for MSC [69].

When we investigated the fSC-SIS constructs, the fSC presented poor integration into 4-layer SIS, which may be due to the matrix microarchitecture that consist primarily of compacted collagen fibrils in densely stacked layers knitted together at production [160]. The manufacturing procedure needed to convert the porcine SIS to an sterile, acellular and dehydrated clinical usable product involves different laboratory processes such as treatment with detergents, enzymes, alcohols and acids or bases [161] This might impact the ECM protein properties and changes the micro-environment to less favourable for cell integration. A study conducted on endothelial cell seeded onto SIS showed cells lining the matrix, and only sparse random integration because of the sterilisation process [162], all in concordance with our findings. Another study described successful integration of rat MSC into SIS (histological data not shown in the article), but the method used slightly differed from our as they injected the cells into the matrix [163]. When we seeded fSC onto the casted collagen gels, the cells integrated, proliferated and were evenly dispersed throughout the gel, suggesting a preferable milieu within the collagen gel for the fSC, compared to SIS. The collagen gel formation is a self-assembling process of monomers forming collagen fibrils, whilst the structural architecture of SIS is organized by specific tissue residing cells creating the ECM with the proper qualities the tissue demands [164, 165]. Our gel consists of collagen type I exclusively, while SIS consists of a mix of collagen type I, III and V, glycosaminoglycans (e.g. hyaluronic acid) and fibronectin among many other molecules [165], giving the gel and the matrix different properties.

It is well known that the cell culture substrate affects the cell properties and may induce differentiation [166], and therefore we wanted to investigate if the cells would turn into more epithelial or endothelial phenotype by keeping the cells in the collagen-PCL constructs in culture for a prolonged time. However, no expression of CD31 (endothelial) or PCK (epithelial) could be detected, indicating that the cell phenotype remained stable. The fSC in the collagen-PCL construct continuously proliferated for up to 6 weeks and there was increasing remodelling of the gel throughout the time points, finalizing at the endpoint at 8 weeks where the top cell-containing collagen layer nearly had disappeared by detachment of collagen from the PCL in this *in vitro* model. This indicated that this was beyond the time limit to keep this construct in culture *in vitro*.

The cells in the collagen-PCL constructs had high and increasing expression of α -SMA compared to cells in SIS, which might be correlated to collagen remodelling. Bildt *et al.* [167] presented that inhibition of the matrix metalloproteinase (MMP) activity of periodontal ligament cells cultured in collagen led to decreased α -SMA expression and collagen gel contraction by the cells. MSC have high MMP activity on the cell surface and collagen degradation activity [168], hence the detected collagen remodelling properties and α -SMA expression by the fSC reinforces their MSC characteristics.

Neo-angiogenesis is vital in the graft area, since without a rapidly formed vasculature there might be an ischemic region without oxygen or nutrient supply, and no waste disposal with following necrosis and graft failure [169, 170]. Therefore, we investigated the angiogenic properties of fSC in a tube forming assay, mimicking the vessel formation of the endothelial cells. The conditioned medium from the fSC slightly increased the number of tubes formed by the HUVECs, indicating some angiogenic properties of fSC.

To conclude this study; foetal cells derived from 2nd trimester subcutaneous tissue demonstrated favourable characteristics for preparation of autologous tissue transplants before birth. Our study supports the theory that cells can be obtained from the foetus during pregnancy for use in tissue engineering after birth.

4.3 AMNION AND MNC ENRICHED PRGF GEL FROM UCB (STUDY III)

The purpose with our last study was to find a non-invasive method to create an autologous tissue graft after birth, ready-to-use at the time for correction. In this study we investigated two highly pro-angiogenic types of autologous constructs made from the non-invasive source: term placenta including; 1) Amnion, and 2) MNC-enriched PRGF gel from UCB. This is, to our knowledge, the first study on fresh amnion and PRGF, combined with a biological graft. Our results demonstrated that the placenta can easily be collected directly after birth and the amnion can be separated from the chorion membrane, combined with a carrier matrix and be ready for use in less than one hour after birth.

We investigated if the properties of the amnion were maintained after being kept in culture. Cell culture conditions such as the medium is *per se* selective for different sub-populations of cells and therefore we compared three different culture media for the amnion. We chose our regular MSC culture medium (MEM-alpha) and compared it with cell culture medium used for amnion epithelial cells (DMEM-EC) and for endothelial cells (DMEM-F12). Strom *et al.* have successfully isolated stem cells from both the amnion epithelial and mesenchymal cell layer using DMEM-EC [171, 172]. They could also show the epithelial stem cell potential by differentiation to all three germ layers; endoderm (liver, pancreas), mesoderm (cardiomyocyte), and ectoderm (neural cells) and the cells were non-tumorigenic when transplanted to mice [107, 173]. DMEM-F12 support growth of several mammalian cells. Yokoo *et al.* isolated human corneal endothelial progenitor cells in DMEM-F12 [174] and Storck *et al.* could show improved proliferation and shorter induction time for differentiation of adipose-derived stem cells [175]. When comparing culture medium, we could not find any significant dissimilarities in morphology or cell viability at any time points despite different culture medium. ADC that had migrated from the amnion was analysed. We noted that cells cultured in DMEM-F12 had a non-fibroblast and non-epithelial morphology whereas when cultured in MEM-alpha and DMEM-EC, the ADC had a fibroblast-like phenotype, and in the flow cytometric assay the ADC cultured in DMEM-EC had highest expression of CD73, and therefore DMEM-EC was used for the forthcoming analyses.

The amnion was combined with a matrix and thereafter analysed. We investigated if the properties of the amnion-residing cells would change over time when combined with SIS, and therefore we kept the constructs in culture. After 21 days of culture, the amnion remained highly viable with a low apoptotic environment and little activation of cell proliferation. Also, the cells present in the amnion when combined with SIS did not alter their phenotype, and both the epithelial and stromal cells had a stable protein expression throughout the study period.

Summary 1 for Study III: The results demonstrate that amnion-SIS construct could be manufactured and in cases of delayed correction of the defect, the constructs could be kept in culture for 21 days, indicating a potential for short-term storage of the construct.

Our study also showed that the PRGF can be produced within 30 min after retrieving the UCB, isolation of MNC took up to one hour with minimal addition of GMP-compliant products, and gel formation occurred within 45 min. In total, a complete MNC-enriched PRGF construct could be prepared within 2–3 hours after birth. At this point the constructs could be used for surgical correction of the defect.

When trying to produce a PRGF gel 24 h after collection of UCB no gel formation was noted. The gel forming process is a controlled induced clotting, which is a rapid response to vascular injury to restore homeostasis *in vivo*. Clotting is normally induced by the extrinsic pathway by tissue factor or contact induced when the blood comes in contact with an artificial surface [176]. Jenny *et al.* performed coagulation studies on whole blood collected with addition of anticoagulants, and found clotting promoted by complement proteins [177]. These findings in combination with the contact induced clotting might explain why we fail to produce a gel 24 h

after collection; the coagulation maybe already had been initiated and the proteins involved activated. Further studies must be performed to verify this data.

Characterisation of the PRGF-SIS constructs showed that the MNC in the PRGF gel maintained a robust protein expression of both CD45 and CD34 over time, as for the PRGF gel without SIS. Increased proliferation was not detected but a small increase in number of apoptotic cells was visible, which might be explained by a loss of PRGF-derived growth factors and cytokines at medium changes through the culture. The gel in the PRGF-SIS constructs slowly degraded and was completely dissolved at day 14. PRGF releases growth factors and cytokines, promote cell recruitment, tissue regeneration, angiogenesis, reperfusion of damaged tissue and reduce apoptosis [34, 41-43], properties that promote tissue formation and wound healing, and could reduce the risk of bacterial colonizations [44]. For these reasons, degradation of the gel within 14 days may not be of any concern in a clinical setting.

Summary 2 for Study III: Amnion and MNC-enriched PRGF gel, after minimal handling, are promising candidates as non-invasive sources for autologous grafts in correction of congenital soft tissue defects.

4.4 UMBILICAL CORD, CHORIONIC VILLI BIOPSIES AND UMBILICAL CORD BLOOD MSC

When starting to realise that AF was an unreliable source, we investigated the potential and potency of other extraembryonic tissues as sources for autologous TE. We conducted a pilot study where we investigated three different isolation methods for UC-MSC. After optimization of the enzymatic digestion method we could isolate cells with all methods. The expanded UC cells had homogenous fibroblast-like morphology and showed a rather good proliferative capacity, expressed MSC surface markers but showed a low ability to differentiate into adipogenic and osteogenic lineages. This study showed that cells could be isolated from UC, but requires procedures based on explant methods or enzymatic digestion [135], which are not preferred in GMP production. UC consists of connective tissue with the purpose to protect the umbilical vessels and is fairly stiff and tough to handle and cut into pieces. Dissociation of the UC into single cells involved a harsh enzymatic treatment while attempting to maintain the cells viable. Another drawback was the long time required for isolation and expansion to obtain adequate number of cells, and the difficulties in maintaining a sterile isolation process. The surgical correction of a soft tissue defect is often performed within a week after birth and at that time we only noted colonies with inadequate number of cells. Therefore, we decided not to investigate UC as a starting material further.

We also performed two rather small studies, investigating the ability to isolate MSC from term UCB and 2nd trimester CVB samples. The studies were unsuccessful since few or no cells could be isolated and we came to the conclusions that 1) MSC was a rare population in UCB and, in our hands, UCB is not a good and reliable source for isolation of MSC, and 2) early gestational CVB-samples did not result in enough cells to use in autologous TE. However, of note was that we started with very small CVB sample sizes (1-3 mm tissue pieces that were left over material from CVB performed with an 18-gauge needle (1 mm \varnothing) for routine analysis) and further investigations on larger tissue sample might lead to different results.

4.5 IN VITRO VS IN VIVO STUDIES

In this thesis we examined and validated the obtained cells and tissue with *in vitro* models, which give the opportunity to observe single processes, without endogenous interference from complex biological systems *in vivo*. One example is the angiogenesis assay used. We chose an *in vitro* tube forming assay instead of the *in vivo* chick chorioallantoic membrane assay because of endogenous proteinase activity in the *in vivo* assay that would influence the results.

Nevertheless, *in vitro* data should be interpreted with caution, bearing in mind that *in vitro* models cannot mimic the *in vivo* processes to full extent. On the other hand, there is the translational story of animals and man; with years of *in vivo* studies which in the end could not be translated to the human systems [178]. For example, the vitamin A-derivative isotretinoin that is highly teratogenic in human and other primates causing malformations at embryonic exposure, but not in mouse or rat models. Investigations showed that primates and rodents have different clearance pathways of the drug; primates had high placental transfer of the drug while rodents had very limited transfer [179]. This could be due to anatomical differences and species various pharmacokinetics and dynamics. Another example is the CD28-specific monoclonal antibody TGN1412 that after preclinical *in vitro* and *in vivo* studies was tested in a phase I clinical trial and caused life-threatening severe adverse events after administration of 0.2 % of the dose that was found to be safe in animals to healthy volunteers [180]. Weeks to months of hospitalisations were required afterwards. This reflects the dilemma in predicting the human response in animal models, even, as in this case, in non-human primates [180]. So, if there is a need for *in vivo* studies, a proper and relevant model must be selected for correct interpretations of human response.

In our settings we can ignore species-dependent variances in pharmacodynamics when pharmaceuticals are not involved in our studies, and investigation of our engineered fSC constructs in an autologous or syngeneic rodent model is feasible. Subcutaneous biopsies could be collected during local anaesthesia and cells could be isolated and expanded. When sufficient number of cells has been obtained, constructs can be produced and transplanted onto the back of the rodent and thereafter validated. In 2014 Kushnaryov *et al.* presented a study on autologous cartilage grafts in a rabbit model. They harvested cartilage from the same animal and expanded the chondrocytes before production and implantation of the graft. This study demonstrated successful implantation and maturation of the autologous cartilage construct. The biomechanical properties were also improved compared to *in vitro* controls [181].

To examine the properties of the other constructs investigated in this thesis *in vivo* would demand a large animal model with reproductive systems that resembles the human anatomy [182, 183] to be able to isolate AF, UC, UCB and placenta for the production of autologous grafts. Kaviani *et al.* isolated AF from pregnant ewes, expanded and seeded the AFC onto a matrix with sustained morphology, suggesting sheep as a prospect *in vivo* model [184]. To overcome the obstacles with the reproductive and anatomical differences, one can use human tissues or cells for the constructs and implant them in immunocompromised mice. For example, Ansari *et al.* isolated human gingival MSC for muscle tissue engineering, loaded the cells in alginate microspheres together with factors for myogenic differentiation and implanted into immunocompromised mice for muscle regeneration evaluation [185]. This methodology using immunocompromised mice has almost become essential for preclinical studies of various diseases and clinical conditions [186-189] and is a useful tool for investigation of infectious diseases [190, 191].

In the end, to be able to bring any of our investigated constructs to the clinic, proper *in vivo* studies must be conducted to ensure absolute safety for the affected neonates diagnosed with congenital soft tissue defects.

5 CONCLUSIONS

To summarise and conclude this thesis, our studies showed that 2nd trimester fSC possessed a high proliferative capacity with a low frequency of senescent cell, demonstrated mesenchymal characteristics, integrated and proliferated in collagen gel. Amnion could easily be isolated and similarly, a MNC enriched PRGF gel from UCB could be produced with minimal handling. They could both be combined with a matrix short after birth.

The UC showed potential for cell isolation, but the expansion took too long time, and it was difficult to maintain a sterile environment during cell isolation.

AF was not a reliable source for mesenchymal cell isolation, CVB might be a good source but it has to be examined with more starting material, and, in our hands, UCB was not a good source for MSC isolation.

Therefore, our main conclusion is that depending on the timeframe from diagnosis to birth, and type, size and severity of the defect, either 2nd trimester fSC, term amnion or MNC enriched PRGF gel are the preferable perinatal tissue sources for autologous TE and correction of congenital soft tissue defects.

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8 ORIGINAL PAPERS I-III